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Evaluation of a New Procedure for Isoamylase Measurement by Selective Inhibition

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Summary: The authors have evaluated a new procedure for the estimation of pancreatic and salivary isoamylase using a new selective inhibitor reagent (Enzamyl Isoamylase, Gödecke[1]), Germany; composition covered by patent No. P 3404876.6).

Provided the conditions of the assay presented here are correctly selected, a standard curve is not necessary and the activities of both isoenzymes can be calculated by a simple formula.

In view of its rapidity and precision, this new procedure appears to be useful as a screening test. It correlates closely with a reference electrophoretic method.

Bewertung eines neuen Verfahrens zur Messung der Isoamylasen durch selektive Hemmung

Zusammenfassung: Die Autoren haben ein neues Verfahren zur Bestimmung von Isoamylasen aus Pankreas und Speichel überprüft, indem sie ein neues selektives Inhibitorreagenz (Enzamyl-Isoamylase, Gödecke[1]) AG, Deutschland, Zusammensetzung gemäß Patent Nr. P 3404876.6) verwendeten.

Bei Wahl der richtigen Versuchsbedingungen kann auf eine Standardkurve verzichtet werden, und die Berechnung der Aktivitäten der beiden Isoenzyme kann nach einer einfachen Formel erfolgen.

Wegen ihrer Schnelligkeit und Genauigkeit erscheint die neue Technik als Screeningtest geeignet. Sie korrelierte sehr gut mit einer elektrophoretischen Referenzmethode.

Introduction

The present study deals with a new procedure for measuring pancreatic isoamylase activity (Enzamyl Isoamylase, Gödecke[1]), Germany; composition covered by patent No. P 3404876.6). This procedure uses a wheat germ selective inhibitor which is already known (1–3), but obviates the need for a standard curve by selecting an inhibitor concentration capable of suppressing 50% of the pancreatic isoenzyme activity and 100% of the salivary isoenzyme activity.

The aim of this investigation was:

(i) to determine the incubation conditions (time, temperature, reagent mixture) required for the operation of the calculation principle proposed by the manufacturer;

(ii) to verify the linearity of the residual activity on reference fluids (saliva, duodenal fluid) after inhibition under the suitable incubation conditions;

(iii) to evaluate the precision of the test;

(iv) to compare the results obtained on sera by this new technique with those given by an electrophoretic method.

[1]) Now: Organon Teknika Medizinische Produkte GmbH, Freiburg im Breisgau.

Material and Methods

Samples

Fifteen duodenal fluids collected by duodenal tubing and forty sera were obtained from patients admitted to the wards. Saliva was collected from fifteen healthy volunteers. Special care was taken to avoid contamination of the duodenal fluid specimen collections by saliva: electrophoresis and blue starch staining were used to assess the purity of each duodenal fluid and each saliva.

α-Amylase activity assay

Serum and duodenal fluid α-amylase was measured on a Gemsaec Centrifugal Analyzer (Electro Nucleonics Inc) at 30 °C by the maltotetraose method (4): intraday precision for repeated assay (n = 15), coefficient of variation less than 2%; interday precision at 2 levels of quality control material run in each series (n = 20), coefficient of variation less than 2.5%; activity in serum sample stable for one week at room temperature and at least 4 weeks at −20 °C; normal serum values up to 42 U/l.

Electrophoresis of α-amylase isoenzymes

Electrophoresis was performed by a modification of the method of *Takeuchi* et al. (5); staining reagent was prepared according to *Davies* (6).

Electrophoresis procedure

Cellulose acetate membranes were placed in 0.02 mol/l phosphate buffer (pH 7.40). After blotting, 3 µl of sample were applied at the mid-point. Samples with high amylase activity were diluted in the same buffer to give an activity around 200 U/l. Electrophoresis was carried out at 4 °C in the same buffer at 300 V for 2 hours.

Staining reagent preparation

Blue starch tablets (Phadebas Amylase tablets) were ground to a fine powder in a mortar and mixed with 5 ml of 2% Agar-Noble per tablet. The mixture was boiled for a further 30 min and then kept at 42 °C.

Staining procedure

At the end of electrophoresis, membranes were blotted, stained by pouring the blue starch solution onto them and kept in an incubator at 37 °C for one hour. After removal of the blue starch gel, the cleaned and dried membranes were scanned in a Beckman integrator (R112−115) using a 0.4 mm · 5 mm slit beam (620 nm).

α-Amylase isoenzyme measurement by the selective inhibition method

Inhibitor solution (Enzamyl Isoamylase, Gödecke[1]), Germany; composition covered by patent No. P 3404876.6) and sample were mixed in equal volumes. After incubation, the residual α-amylase activity was measured and the results calculated as follows:

1. pancreatic isoamylase activity (U/l) = residual activity · 4, where 4 represents the sample/inhibitor predilution (· 2) and the 50% inhibition of pancreatic isoenzyme;
2. salivary isoamylase activity (U/l) = total activity − pancreatic activity.

Results

Validation of experimental conditions and calculation procedure

Figure 1 illustrates the relationship between inhibition and the preincubation time at 30 °C. For each of the 3 fluids maximal inhibition was achieved or nearly

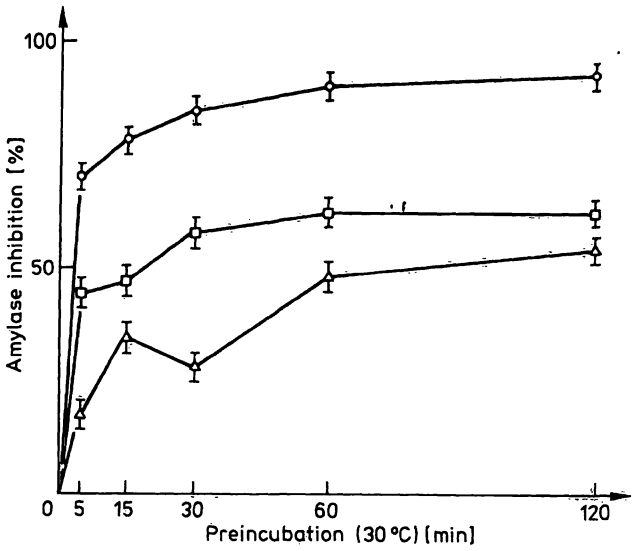

Fig. 1. Fraction of amylase inhibition (±SEM) as a function of preincubation time at 30 °C in 5 saliva (o−o), 5 duodenal fluids (Δ − Δ) and 5 sera (□−□).

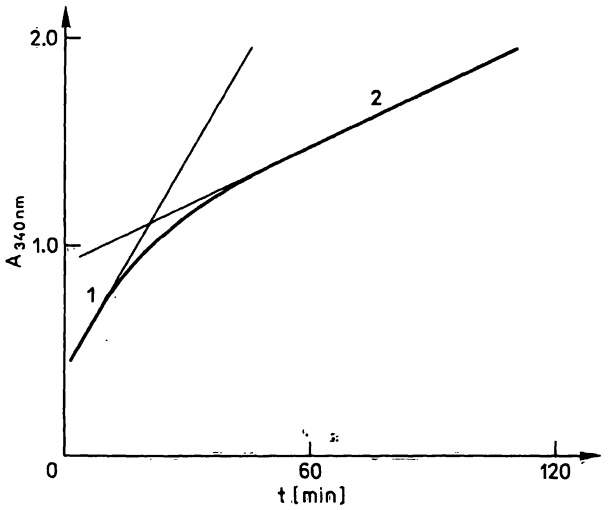

Fig. 2. Time course of a one-step procedure assay: (1) first slope corresponding to the initial rate; (2) second slope corresponding to the activity remaining after inhibition.

achieved at a preincubation time of 60 min onwards: more than 90% for saliva, around 45−50% for duodenal fluid and 60% for serum.

The temperature of preincubation does not influence the result; no difference was found between experiments performed at 25 °C and 37 °C; however results obtained at 25 °C were more scattered owing to the low residual activities (approaching the detection limit) recorded at that temperature.

Figure 2 presents the results of a continuous assay where 50 µl of inhibitor, 50 µl of serum and 1 ml reagent were mixed at once and the kinetics recorded

on a spectrophotometer Beckman (model 25). In 3 sera, total activity was estimated from the extrapolation of the initial rate and residual activity after inhibition from the second slope. These results were compared with those obtained with the same sera by a two-step procedure. This comparison reveals that neither total activity nor maximal inhibition could be correctly estimated by the one-step procedure and that their reliable determinations require preliminary measurement of total activity and a one hour preincubation with the inhibitor.

Figure 3 shows the residual activities recorded when a same volume (50 µl) of sample (duodenal fluid or saliva) was preincubated with increasing volumes of the inhibiting solution. This graph reveals that a 50% of pancreatic amylase activity was only obtained for a 1/1 (v : v) sample-inhibitor ratio.

Another set of 3 experiments was devised to validate the calculation procedure. One aliquot of duodenal juice and a second one preincubated for 1 hour with the inhibition solution (1 : 1) were submitted to electrophoresis; after staining, the electrophoretic spots were scanned in a direction perpendicular to the line of migration (fig. 4). The results show that the ratio between the areas of initial and residual activities ranged from 77–23% to 83–17% and thus came close to the 80–20% ratio expected from the theoretical calculation.

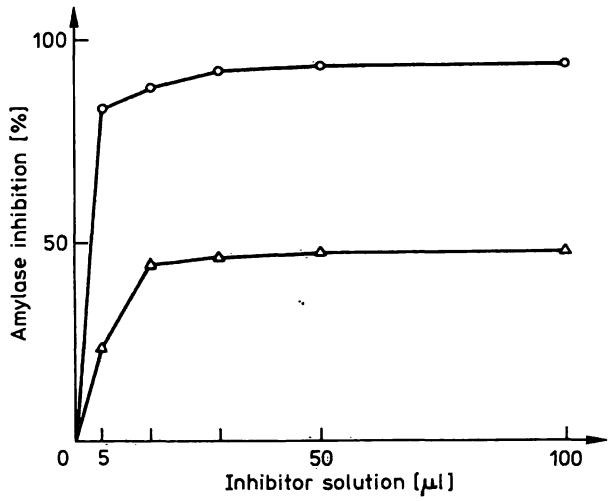

Fig. 3. Evolution of amylase inhibition in salivary (o–o) and duodenal (Δ – Δ) fluid (50 µl of each) as a function of the inhibitor solution volume.

Linearity of reaction

Total and residual α-amylase activities measured in saliva and duodenal fluid under the preincubation conditions selected are plotted in figure 5 against the corresponding dilutions. The assay is linear up to 350 U/l for total activity and at least up to 200 U/l for residual activity after inhibition. It must be noticed that in the case of salivary amylase, inhibition was never complete: above 100 U/l total activity, a 5% residual activity was always observed.

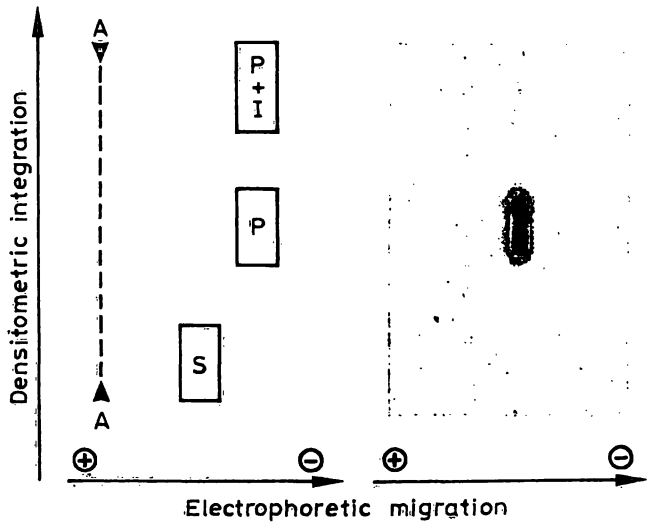

Fig. 4. Experimental design to validate the 50% inhibition of pancreatic isoamylase by an electrophoretic method (S = saliva; P = duodenal fluid; P + I = mixture of duodenal fluid and inhibiting solution; A = application).

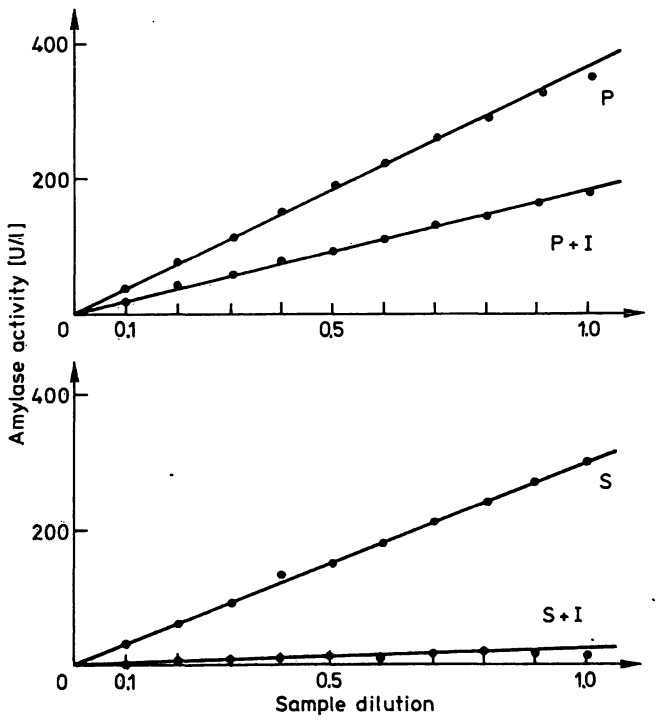

Fig. 5. Evaluation of total and residual α-amylase activities in saliva and duodenal fluid as a function of sample dilution (S = saliva; P = duodenal fluid; S + I and P + I = mixture of saliva or duodenal fluid and inhibiting solution).

Between assays precision

Twenty sera were repeatedly estimated in 6 assays: the coefficients of variation never exceeded 2% for total amylase activities and increased up to 3.6% for residual activities above 10 U/l and to 12.5% for still lower values approaching detection limit. It must however be noted that even in these last cases, the standard deviation of between assays precision remained as low as 0.5 U/l.

Comparison to a reference method

In figure 6 the values obtained from 22 sera by the selective inhibition technique (ordinates) are plotted against the corresponding values given by the electrophoretic method (abscissa). The formula of the regression line was $y = 1.02\,x + 2$ and its slope was not significantly different from the identity line $\beta = 1.00$ ($t = 0.6338$, $p > 0.50$); moreover the standard deviation from regression was equal to 10 U/l (*i. e.* a CV of 12% for mean value). These features emphasize the similarity of results provided by both methods.

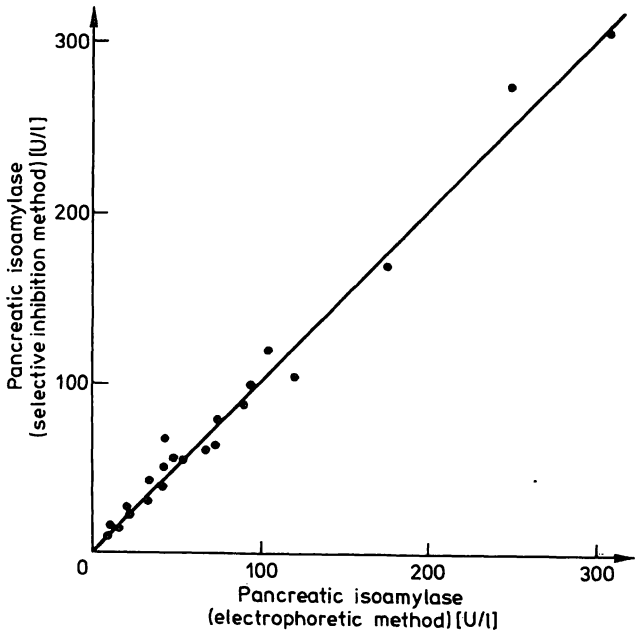

Fig. 6. Correlation between the electrophoretic method and the selective inhibition method for measuring pancreatic isoamylase activity ($y = 1.02\,x + 2$; $r = 0.9923$; $n = 22$; $p = 0.001$).

Discussion

The present evaluation based upon the use of reference fluids reveals that the type of calculation forwarded by the manufacturer to estimate the contents in pancreatic and salivary amylase activities of biological fluids is valid providing following conditions are fulfilled:

- the use of a two-step procedure with determination of total activity in one aliquot, and of residual activity after selective inhibition in a different aliquot.
- a 1/1 (v : v) ratio between sample and inhibiting mixture;
- a one hour incubation of sample and inhibiting mixture before measuring residual activity;
- a residual activity after inhibition greater than 5% of total activity.

The last condition is based on the fact that inhibition of salivary amylase never exceeded 95% of total activity. Application of the calculation formula for total activity concentrations greater than 300 U/l would thus provide a value above 30 U/l for the pancreatic component even in the case of a hyperamylasaemia of pure salivary origin.

Applied to sera taken at random, this technique has provided results superimposable on those obtained with an electrophoretic reference method.

The repeatablility of the technique is quite satisfactory except for very low residual values that can only be observed with fluids displaying a poor content of pancreatic amylase and a high content of the salivary isoenzyme. However, it must be noted that this assay is primarily designed to estimate with precision the pancreatic component in hyperamylasaemia of doubtful aetiology, a situation that is often encountered when amylasaemia rises two- or three-fold above the upper normal limit (7, 8). In this situation, the new assay may be suggested as a suitable screening test, in view of its linearity, its repeatablility and its simplicity linked the fact that a standard curve is not needed. Nevertheless, a careful evaluation of this assay in pancreatitis attacks is still to be made, in order to assign its place among the other diagnostic tests. Indeed it must be remembered that simultaneous determination of amylase and pancreatic lipase in plasma by rapid and accurate methods has already achieved a very high level of confidence for the detection of acute or relapsing pancreatic disorders (7, 8).

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